Prof. Roger M. Loria
loria@hsc.vcu.edu
Virginia Commonwealth University, Medical Center
Microbiology, Immunology, Pathology and Emergency Medicine
1101 E. Marshal Street
Richmond, VA
232980678
Phone: 804-828-9717
Fax: 804-828-5862

Project Title: Preliminary Studies on the Effects of Androstentriol Induced Immunomodulation in the Treatment of Traumatic Shock
ONR Award No: N00014-03-1-0362
Reporting Period: June 1, 2004 - May 31, 2005
Award Period: January 30, 2003 - September 30, 2005

Scientific and Technical Objectives

Specific Aim 1: Examine the ability of androstenetriol to improve survival in a rodent model of combined hemorrhage and tissue injury (traumatic shock).

Hypothesis 1: The use of androstenetriol shortly after the onset of traumatic shock will improve survival in a rodent
model of combined hemorrhage and tissue injury (traumatic shock).

Specific Aim 2: Examine the ability of androstenetriol to modulate the immune and inflammatory response systemically and in multiple organ systems in response to traumatic shock.

Hypothesis 2: The administration of androstenetriol shortly after the onset of traumatic shock will result in changes in the immune and inflammatory response systemically and in individual organ systems which will assist in understanding the mechanisms by which androstenetriol may improve outcome.

Specific Aim 3: Examine the ability of androstenetriol to preserve microcirculatory flow and tissue oxygenation during the early stages of traumatic shock

Hypothesis 3: The use of androstenetriol shortly after the onset of traumatic

shock will be associated with improvements in microcirculatory flow and tissue oxygenation.

Approach

A preclinical model of severe traumatic shock incorporating key injury elements including hemorrhage, tissue injury, and transfusion with packed red blood cells will be used to test the hypothesis that immunomodulation with androstenetriol after injury will improve survival. Using state of the art technology, systemic and organ specific cytokine and inflammation profiling in the model will allow for an understanding as to how androstenetriol modulates the immune and inflammatory response to injury.

Concise Accomplishments

Two rodent models, the first (n=24) consisting of laparotomy (soft tissue injury) and 40% hemorrhage of total blood volume. 60 minutes later, they were resuscitated with crystalloid fluid and packed red blood cells and observed for three days. Twelve animals received AET with 100% survival; in the untreated control group (n=12) mortality was 25%, ($P < 0.04$, Barnard's unconditional test of superiority using difference of two binomial proportions). In the second model (n=29), animals were hemorrhaged down to a 35-40 mmHg MAP, resuscitated as described earlier and observed for two additional days. Mortality in the untreated group (n=11/16) increased to 68.75%. Nevertheless, AET treated animals (n=13) had a mortality rate reduced to 30.76% (4/13). Kaplan-Meier analysis yields a P Conclusion: The results show that AET provides a significant protective effect and improves survival in two different studies of severe trauma-hemorrhage and shock.
Immunological measurements of chemokines and cytokines responses were performed using Rat Cytokine/Chemokine kits which enables simultaneous measurement of fourteen rat cytokine/chemokines.
Results: AET treatment markedly alters the levels of chemokine and cytokines and modulates the inflammatory response.

Expanded Accomplishments

This study utilizes a unique and clinically relevant model of traumatic shock which optimizes the transitions of the preclinical results to clinical studies. It includes the attributes of tissue injury and hemorrhage, avoidance of general anesthesia, provision of clinically relevant analgesia, and provision of packed red

blood cells as opposed to whole blood. The avoidance of general anesthesia in models of traumatic shock and resuscitation may be of particular importance when testing immune modulation as a treatment strategy for critical illness and injury and models which replicate the clinical setting with a high degree of fidelity are likely to yield results that are more definitive.

The major goals of this project have been accomplished and illustrate that immune modulation is a feasible and rational approach for the treatment of hemorrhage-trauma and shock. The results demonstrate that a single injection of androstenetriol is 100% protective in the 40% blood loss trauma shock model, $P < 0.04$. However, this model was associated with relative low mortality 25%, and therefore we proceeded in developing and testing the effectiveness of androstenetriol in a more severe model. When attempting to augment the volume of blood loss above 40%, in order to increase mortality above 25%, there was a high variability in survival. Consequently, a model using 35-40 mmHg mean arterial pressure as end point to determine the level of blood loss was used. On average this resulted in about 60% blood loss (43-65%) with an increase in mortality to 70%. The results, unequivocally show that mortality was almost 3 times greater in vehicle treated animals as compared to AET treated ones,(11 vs 4).The overall protective effects of AET in both hemorrhage-trama shock models are presented in figure 1.

The results provide a strong basis for the support of an effort to initiate a clinical trials process.

Several factors influenced the immunological results. These included the low numbers of available samples, due the high mortality of vehicle treated animas. A large biological variability of animal response to hemorrhage trauma and shock as well as the variability of the multicytokine assay. Nevertheless, the immunological studies show that survival is associated with increase levels of certain chemokine/cytokines and that AET has an effect on specific cytokines. Chemokine and cytokines measurements were done in both hemorrhage-trauma shock models. The data from the 40% blood loss model (volume) show increase level of GRO/KC (growth-related oncogene) CXC-chemokines which attract neutrophils and activated T lymphocytes. Indeed the level of GRO/KC is significantly increased in survivors as compared to baseline-untreated animals,$P= 0.04$; or to the level observed in vehicle treated at 24 hr, $P=0.02$. The level of MCP-1(monocyte chemo attractant protein-1)stimulates expression of the cell surface antigens (CD11c, CD11b) and the expression of cytokines (IL1,IL6). MCP-1 is also a potent activator of human basophils. In survivors the levels of this chemokine was 1336 ± 437.36 pg/ml as compared to 105.55 ± 18.92 pg/ml, in baseline untreated hemorrhaged animals, $P = 0.001$. In additon, the levels of IL-1 for vehicle treated animals surviving 72 hrs was 56.99 pg/ml, considerably below the baseline level, and significantly lower than the level of AET treated animals, 181.37 pg/ml, $P=0.001$. The latest observation is of

particular interest since the data show that AET is able to restore the levels of IL-1 to baseline levels while vehicle treated animals do not. These observations are also confirmed and extended, Figure 2, when AET injected 24 hrs prior to a 60% blood loss and trauma. AET mediates a marked reduction of the inflammatory cytokines IL-1 alpha, IL-1 beta, IL-10 and TNF alpha. Injection of AET 24 hrs prior to the hemorrhage trauma procedure, did not have a significant effect on the baseline (zero time after the procedure)levels of these cytokines . Conclusion: androstenetriol markedly reduced the excessive inflammatory response associate with hemorrhage - trauma and shock.
A muscle crush apparatus, was designed and developed. The drawing are included, Figure 3. Muscle crush injury may be incorporated with this trauma hemorrhage shock model in the future.

Uploaded Files:
AET cytokine levels.ppt
CrusherDrawings.pdf
hemorrhage trauma PNRS poster.ppt.ppt
Mortality data.ppt

Work Plan

The work plan for the next two months will advancing the immunological studies. Time permitting, we will perform analysis of cytokines at specific time points, irrespective of the survival outcome in the more severe hemorrhage trauma model- 60% blood loss. We are in the process of completing the first manuscript entitled "Effects of Androstrenetriol in a Rodent Model of Combined Hemorrhage and Tissue Injury" to be submitted. Two other manuscripts will be prepared for publication.

Problems/Issues

Animal problems – we continue to be plagued by the so called " rat respiratory virus "(see
http://www.radil.missouri.edu/RADILinfo/research/ResearchRRV.
asp for additional details). Our new attending veterinarian (Dr. Bobby Collins) is working closely with us to monitor the situation . Room sterilization was implemented, including the use of filter top cages, protective garments for all animal handlers, and ventilated cage racks and a laminar flow cage-changing station. During this report period 75 surgeries were performed, of these we had 25 cases of animals which could not be included in the protocol, mostly because

of the problem mentioned above.
The use of the multiplex chemokine/cytokine assays which measured simultaneously 14 different rat cytokines was not straight forward. We tested the Biorad B plex cytokine kit and the Lycoplex plate system. Both systems proved to have problems making the analysis of the data difficult. Only more testing and additional experience will permit us to determine the better system. There is considerable variability in the cytokine levels after hemorrhage-trauma prior to any treatment. In addition, commercially available anti-RAT antibodies are not as well tested or available as either mouse or human antibodies. These factors make the evaluation of the cytokine results markedly more difficult to evaluate. The complexity of the hemorrhage trauma model, and our limitation to perform only one surgery per day, or 4 per week, and the requirements to randomizing AET treatment or vehicle, made it difficult to obtain the correct matching number of samples as long as survival experiments were being performed. With our ability to concentrate on the immunological experiments we should be able to obtain better samples.

Peer-Reviewed Journal Articles

Status	Text

Books or Book Chapters

No book or book chapters reported.

Technical Reports (Non-refereed Publications)

No technical reports reported.

Abstracts/Presentations/Posters/Conference Proceedings

12th Annual Meeting of the Psychoneuroimmunology Research Society Denver, Colorado • June 9-11, 2005 • Brown Palace Hotel
Presentation at the 2004 ATACCC conference St. Petersburg, Florida
Abstract presentation at the 2005 ATACCC conference - St Petersburg, Florida.

Poster presentation at the ATACCC conference - St Petersburg, Florida.

Awards/Honors/Invention Disclosure

No awards/honors reported.

Patents Submitted

Provisional application entitled " Methods for treating shock " was filed with the PTA office.

Patents Issued

No patents issued reported.

Technology Transfer

No technology transfer reported.

ONR Database Statistics

Use of Human Subjects	No
DoD Personnel Used	
Use of Animals	Yes
Animals Used	Other
Use of Recombinant DNA	No
Degree(s) Granted	0

PI/CoPI Information

0 PI/CoPI Minority Women**

0 PI/CoPI Minority Women

0 PI/CoPI Non-Minority Women

0 PI/CoPI Minority Men**

2 PI/CoPI Non-Minority Men

	Minority	Non-Minority	Total
Women	0	0	0
Men	0	2	2
Total	0	2	2

Post Doctoral Information

0 Post Doctoral Minority Women**

1 Post Doctoral Non-Minority Women

0 Post Doctoral Minority Men**

0 Post Doctoral Non-Minority Men

	Minority	Non-Minority	Total
Women	0	1	1
Men	0	0	0
Total	0	1	1

Grad Students Information

0 Grad Students Minority Women**

2 Grad Students Non-Minority Women

0 Grad Students Minority Men**

0 Grad Students Non-Minority Men

	Minority	Non-Minority	Total
Women	0	2	2
Men	0	0	0
Total	0	2	2

Undergrad Students Information

0 Undergrad Students Minority Women**

0 Undergrad Students Non-Minority Women

	Minority	Non-Minority	Total
Women	0	0	0

0 Undergrad Students Minority Men**	Men	0	0	0
	Total	0	0	0
0 Undergrad Students Non-Minority Men				

Publication Totals

Total Number of Peer-Reviewed Journal Articles:	0
Total Number of Books or Chapters:	0
Total Number of Technical Reports:	0
Total Number of Abstracts/Presentations/Posters/Conference Proceedings:	4
Total Number of Patents Issued:	0
Total Number of Patents Pending:	1

** *Under-represented or minority groups include Blacks, Hispanics, and Native Americans. Asians are not considered an under-represented or minority group in science and engineering.*
*** *Supported at least 25% this year on contract/grant.*

Other Sponsored Work

TITLE	SPONSOR	FUNDING	START DATE	END DATE
Modulation of oxygen debt to survive blood loss	Defense Advanced Research Project Agency (DARPA)	1,299,998.00	January 1, 2005	December 31, 2006

Foreign Collaborations

No foreign collaborations reported.

Uploads

MET cytokine levels.ppt
CrusherDrawings.pdf
Mortality_data.ppt
hemorrhage trauma PNIRS poster.ppt.ppt
overview-2.ppt

Figure 1

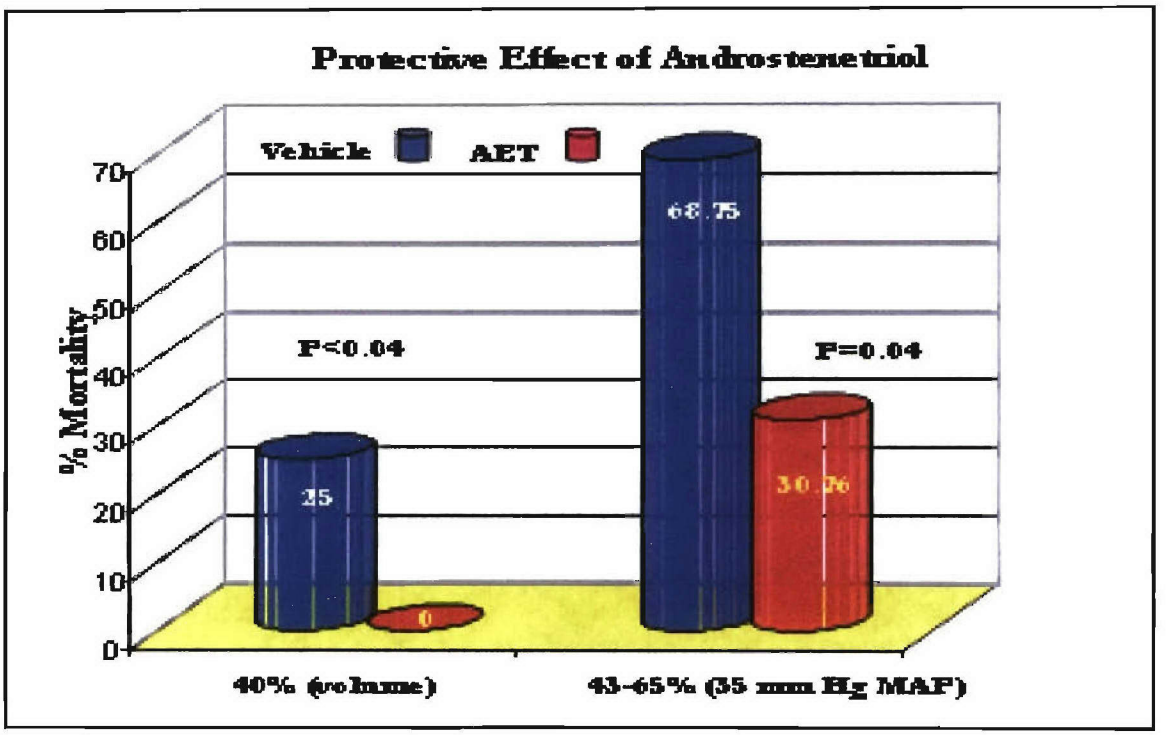
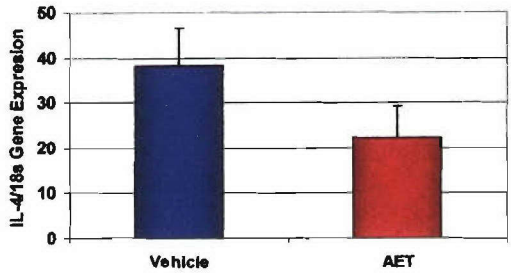
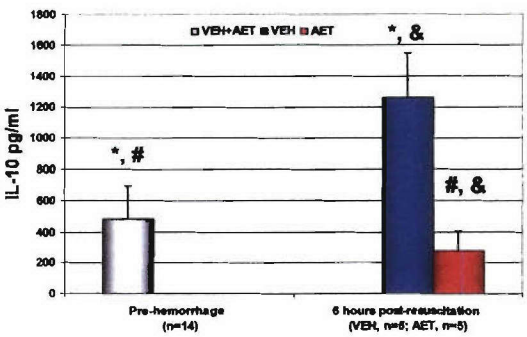

Protective Effect of Androstenetriol
Vehicle
AET
% Mortality
70
60
50
40
30
20
10
0
P<0.04
P=0.04
25
0
68.75
30.76
40% (volume)
43-65% (35 mm Hg MAP)

Figure 2

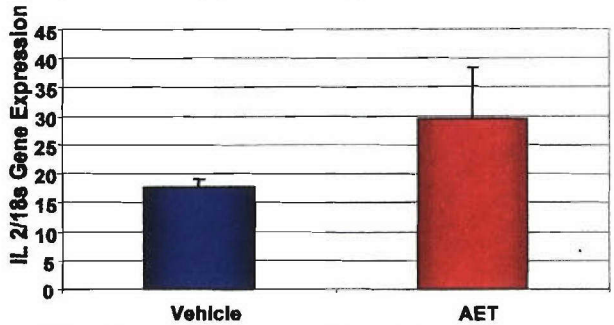
AET MODULATION OF TH2 and TH1 CYTOKINES



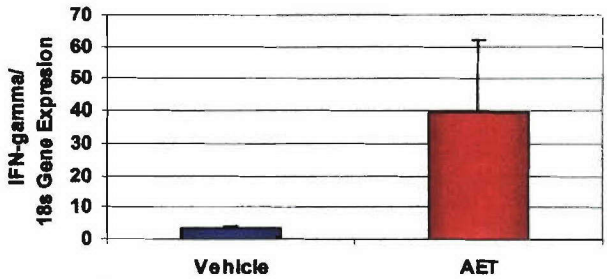
Reduction of IL-4 Gene Expression in the Spleen Tissue, 48 hours post-resuscitation. AET vs. VEH, p<0.03, Independent Sample Test.



*** VEH 6 hours post-resuscitation/pre-hemorrhage, p<0.003.**
& AET vs. VEH at 6 hours post-resuscitation, p<0.002, Independent Sample Test.
 # AET 6 hours post-resuscitation/pre-hemorrhage, p<0.027.



IL-2 Gene Expression in the Spleen Tissue, 48 hours post-resuscitation
AET vs. VEH, p<0.05, Independent Sample Test.



IFN-γ Gene Expression in the Spleen Tissue, 48 hours post-resuscitation
AET vs. VEH, p<0.11, Independent Sample Test.

Marcu AC et al. J Trauma 2006

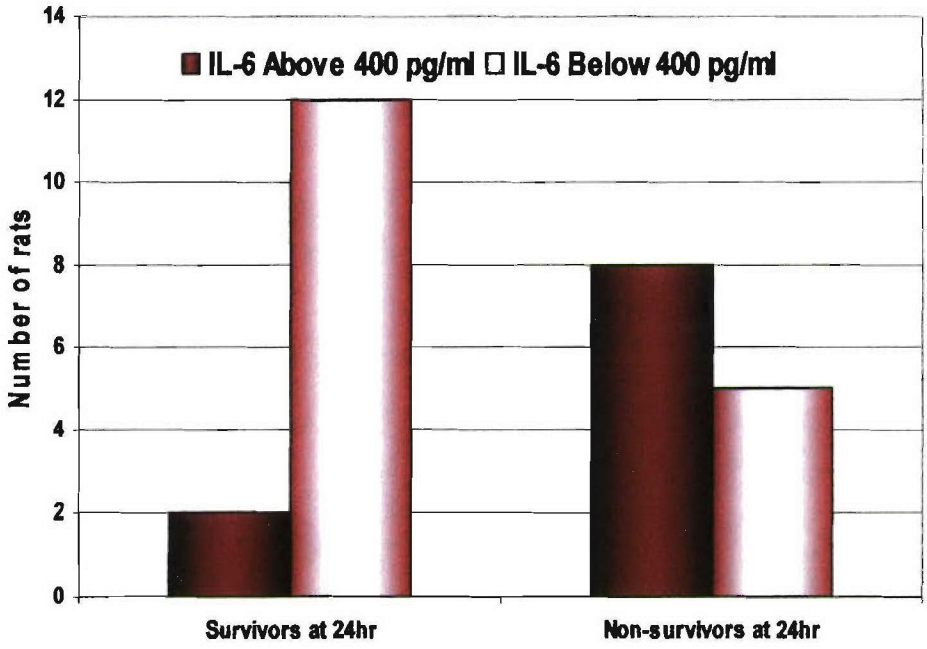
Figure 3

Interleukin 6 (IL-6) as a Prognostic Factor

IL-6 Threshold Levels at 6 hours after Trauma-Hemorrhage Predicted the Differentiation in Survivors and Non-Survivors at 24 hours.

Chi-square analysis

Degree of freedom: 1
Chi-square: 6.45
$p \leq 0.025$.



Androstenetriol Immunomodulation is effective for the Treatment of Traumatic Shock .

Marcu AC., Barbee WR., Ward KR., and Loria RM.

Objective: Test if Androstenetriol Improves Survival in a Rodent Model of Combined Trauma Hemorrhage and Shock.

Approach: A preclinical model of severe traumatic shock incorporating tissue injury, hemorrhage and resuscitaion was used to test the hypothesis that immunomodulation with androstenetriol after injury will improve survival.

Accomplishments: The results indicate that a single dose of AET provides a significant protective effect and improves survival in two clinically relevant models of traumatic hemorrhagic shock.
AET protective effects are associated with an elevation of Th1 and reduction of Th2 cytokines.

VIEWGRAPPH